nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence; and then

(g) amplifying the nucleic acid present in the template via the PCR using the third PCR primer and the plurality of fourth PCR primers[; whereby] <u>under conditions wherein</u> the third primer binds to the sequenced portion of nucleic acid from step (e), and a subset of the plurality of fourth primers binds to the template at locations removed from the third primers such that DNA regions flanked by the third primer and the fourth primer are <u>specifically</u> amplified.

REMARKS

The amendment to the specification at page 26 is to update the status of the allowed application recited therein. The paragraphs added at page 26 are found in the cited Senapathy patent at col. 4, lines 15-50, and col. 5, lines 17-28. A copy of U.S. Patent No. 5,994,058 is attached hereto as Exhibit D.

Claims 1, 12, and 19 have been amended herein. Support for the amendment can be found in Claims 1, 12, and 19 as originally filed. No new matter is added.

Claims 1-29 remain in the case.

A full set of "clean" claims as they presently stand is attached hereto, along with a separate listing of Claims 1, 12, and 19 as amended herein.

Favorable reconsideration is respectfully requested.

Rejection Under 35 USC §112, First Paragraph:

Applicants respectfully traverse this rejection in light of the following comments and the Rule 132 Declaration of Periannan Senapathy, submitted herewith.

In support of this continued rejection, the Office cites to *Genentech v. Novo Nordisk A/S*, 108 F.3d. 1361, 42 USPQ 1001 (Fed. Cir. 1997). In particular, the Office cites with emphasis the following passage from the Genentech case:

> However, when there is no disclosure of any specific starting material or any of the conditions under which a process can be carried out, undue experimentation is required; there is a failure to meet the enablement

requirement that cannot be rectified by asserting that all the disclosure related to the process is within the skill of the art.

Applicants respectfully submit that the above passage is inapposite to the present case because the specification itself discloses all that is required by a person of skill in the to practice the invention without having to resort to undue experimentation. For the Examiner's convenience, a copy of the *Genentech* case is attached hereto as Exhibit A.

The *Genentech* case itself is not particularly relevant to the present situation because the facts of the *Genentech* matter are very different than the facts present in this application. The claim at issue *Genentech* was directed to a method to produce a specifically-defined product, a protein consisting essentially of amino acids 1-191 of human growth hormone (hGH). In contrast to the present specification, however, the dearth of information contained in the Genentech patent specification clearly did not support the breadth of the process claim. Specifically, while the claims in Genentech recited a process to produce hGH via a fusion protein route, the specification did not "describe in any detail whatsoever how to make hGH using cleavable protein expression." Additionally, the specification did not "describe a specific material to be cleaved or any reaction conditions under which cleavable fusion expression would work." *Genentech*, 108 F.3d at 1365.

Moreover, Genentech's claims were drawn to *human* growth hormone, while the prior art cited by Genentech in support of its claims was drawn to *non-human* examples. *Id.*

Lastly, Novo Nordisk successfully argued that at the time of Genentech's filing date, trypsin had only been used to digest proteins in a general fashion, not to cleave a fusion protein in a specific and precise manner to yield an intact and useful protein product, nor had Genentech included a single amino acid sequence out of the "virtually infinite range of possibilities" that would yield hGH in a useful form when cleaved from the fusion protein. *Id.* at 1366-67.

Thus, in the final analysis, Genentech's patent specification failed in virtually every detail to define the parameters of the invention *as claimed*. The same, however, cannot be said of the present specification.

For example, regarding the source of the DNA for analysis, DNA is DNA regardless of the source from which it is derived. Once removed from the source organism, all DNA is the same from a chemical perspective. All DNA contains the same purine and pyrimidine

bases and a sugar backbone linked by phosphodiester bonds. Further still, all DNA functions in the same manner, regardless of its origin. In support of the clear universality of DNA as a carrier of genetic information, Applicant submits herewith as Exhibit B page 42 of James D. Watson's book "Recombinant DNA." There, Dr. Watson states that:

> By now [1983] the initial expectations that the genetic code or chromosomal DNA would prove to be universal have been rigorously confirmed in a large variety of organisms, ranging from the simplest prokaryote to the most complex eukaryote.

Isolating DNA is well known to those skilled in the art and need not be described in any detail in the specification. That this is the case is candidly stated in the specification itself at page 19, line 23-27. The specification goes on to note that "the invention functions with equal success using nucleic acid from any source, including eukaryotic, prokaryotic, animal, plant (both monocot and dicot), fungi, algae, and virus nucleic acids, DNA and RNA included." *Id.*

Indeed, DNA of all types can be purchased commercially from innumerable international suppliers. For instance, genomic human male DNA and genomic human female DNA is available commercially from Promega Corporation, Madison, Wisconsin (catalog nos. G147 and G152, respectively). Promega also sells genomic mouse DNA (catalog no. G3091) and genomic *S. cerevisiae* DNA (cat. no. 3101). Stratagene Corporation, of La Jolla, California, sells genomic DNA from avians, baboons, bovines, canines, cephalopods, chickens, felines, fish, fungi, gorillas, guinea pigs, hamsters, horses, humans, insects, lobsters, marsupials, monkeys, mice, nematodes, plants, porcines, rabbits, rats, salamanders, xenopus, and yeast. See www.stratagene.com and enter "genomic DNA" in the search box. In short, locating a source for target DNA is a very simple means.

This brings us to the clear distinction between the holding in the Genentech case and the present application: whereas Genentech was drawn to a method to produce a particular protein having a desired functionality, the present invention is drawn to an analytical method. The ultimate data generated by the test is not the issue. The issue is whether the specification provides sufficient detail so that *one of ordinary skill in the art* can practice the invention. Here, the relevant audience must be acknowledged, a fact that Applicant respectfully submits has been given little or no weight by the Office. Yes, a patent specification must enable the

invention as claimed, but the specification need not disclose what is well known in the art in order to do so. *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 1385 USPQ 81, 94 (Fed. Cir. 1986).

The present claims recite prior art methods combined in a novel way to arrive at a new analytical method. The individual manipulations comprising the method, such as hybridizing primers and performing the PCR, are undeniably well known to those skilled in the art. The Office noted in its earlier action that the specification makes reference to prior art methods, yet the specification does not teach how these methods are to be modified for use in the present invention. The prior art methods do not have to be modified or adapted in any way to practice the claimed invention.

Looking to the specification itself, the document carefully documents the prior art means needed to practice the claimed invention, including citations to prior papers and patents where appropriate. For example, at page 1, the specification lists no less than seven (7) techniques currently in use for isolating genes and a full citation for each method. The listed methods are cDNA selection, exon trapping, CpG island identification, hybridization using genomic fragments as probes against cDNA libraries, cloning and sequencing of genomic DNA followed by computer analysis of the possible coding regions, Alu-splice PCR, and Alu-promoter PCR. Thus, one of skill in the art is given a rather extensive bibliography of the prior art methods of identifying and analyzing genes and therefore can also be imputed to have knowledge of the shortcomings of these methods. A brief discussion of these shortcomings is presented in the specification at pages 2-3.

Regarding the PCR, this reaction, as noted above, is undeniably well known and extensively practiced in the art. Note also, however, that the specification explicitly mentions that two patents that disclose the PCR process: U.S. Patent Nos. 4,683,195, and 4,683,202. Thus, the practice of the PCR is clearly set forth in the specification by citation to an earlier U.S. Patent.

The PCR reactions are assembled as described in the specification and run in standard fashion. Note that primer concentration is explicitly discussed at page 4, lines 5-19:

> The current invention, however, uses a number of randomized nucleotides linked to the splice junction consensus sequence, with each of the primer species in the primer preparation having a full complementarity with a

particular splice junction present at a unique location in the whole genome. Approximately 500,000 to 1,000,000 exons are expected to be present in the whole human genome for a total estimated 100,000 genes. A sequence with ten randomized nucleotides will have 4^{10} (*i.e.*, 1,048,576), different possible sequences. Thus, a 3' splice junction consensus sequence linked to ten additional bases of randomized sequence (*i.e.*, N_{10} where N is A, C, T, or G) will bind to approximately one million different but uniquely specific splice junctions in the human genome. **By increasing the concentration of each of the primer species in the primer preparation many thousand fold, it has been found that standard PCR amplification proceeds normally. For example, using a 5000-fold increase in primer concentration in a standard PCR reaction, genomic DNA can be amplified specifically so that only the exons present within the genomic DNA are amplified.**

The specification also candidly acknowledges that the method might not amplify every single target sequence due to the extremes in Tm that may be required for their amplification, but that the method will amplify a considerable number of target sequences. See page 5, lines 1-6. Melting temperature is addressed at page 23, lines 25-28:

> The T_m of 10-base sequence with 50% GC content is 30°C, whereas that of a 16-base sequence is 48°C (computed using 2°C for each A and T, and 4°C for each G and C; *i.e.*, $\Delta T_m = 4°(G+C) + 2°(A+T)$). Non-specific binding can be reduced at higher temperatures of annealing.

Note that the formula given for calculating T_m is a very widely employed rule of thumb. Thus, the specification directly addresses the issues of primer concentration and the T_m of both the primers and the target sequences.

At page 9, starting at line 3, the specification discusses how the method is to work when the desired target sequences are shorter than the optimal length for standard PCR amplification. Here, additional randomized base--pairs are added to the primers, so as to make them long enough to yield specific amplification in the PCR. The specification explains the benefits of doing so at page 9, lines 7-29:

> For example, the 8 nucleotide consensus sequence of the 5' splice junction forms a sub-optimal primer length (optimal primer lengths conventionally falling within the range of about 10 to 30 nucleotides), and is not useful as a conventional primer at standard stringent temperature of annealing in a standard PCR reaction. Either the temperature of annealing has to be lowered considerably, which will lead to significant non-specific binding, or the primer may not bind efficiently at the standard temperature of melting. This problem of sub-optimal length is even more apparent for promoter signals (5 to 6

nucleotides) and polyA signals (6 nucleotides). Increasing the length of the consensus sequence primer by adding a few randomized nucleotides imparts several advantages: 1) It increases the length of individual primers in the primer cocktail to a standard primer length. Each of the signal sequences in the genome represented by its consensus sequence and its flanking sequences, together comprising the standard primer length, will have a fully complementary primer species within the primer preparation. Therefore, each full-length primer species within the primer cocktail is a stand-alone primer for any given consensus sequence at a particular or unique location present in a template nucleic acid. Because the length of the primer is increased, a standard melting temperature can be employed in the PCR reaction. 2) It increases the specificity and binding affinity of individual primers contained in the primer preparation when each of them binds to a different binding site in the sample DNA. A mammalian genomic DNA sample contains perhaps one million different 5' splice junctions, each of which is of about 8 base pairs in length and which is identified by a consensus sequence. However, by adding randomized nucleotides to the consensus sequence, all the different possible sequences (of the length of the randomized nucleotides) adjacent to the signal consensus sequence, for example, at the 5' splice junction, are generated in the primer preparation, thereby providing an individual primer species with full sequence complementarity to each of the 5' splice junction sequences and a part of its flanking sequence within the DNA sample.

Cycling temperate, re-annealing temperature in particular, is addressed in the paragraphs inserted at page 26.

Thus, the specification provides all the information required to take a known method, namely the PCR, and extend it to practice the present invention as broadly as it is claimed. Critical parameters such as primer concentration, T_m, target sequence length, and cycling temperature are specifically addressed. The source of the DNA to be analyzed, as noted above, is explicitly stated in the specification as being irrelevant. The method will function with DNA from any source.

Any remaining considerations are routine in the art and need not be discussed in any great detail in the specification. A cite is provided for such routine considerations, knowledge of all of which clearly can be imputed to the ordinarily skilled molecular biologist:

> Many of the steps noted below for the manipulation of DNA, including digesting with restriction endonucleases, amplifying by PCR, hybridizing, ligating, separating and isolating by gel electrophoresis, transforming cells with heterologous DNA, selecting successful transformants, and the like, are well known and widely practiced by those skilled in the art and are not extensively elaborated upon herein. Unless otherwise noted, the DNA protocols utilized

herein are described extensively in Sambrook, J., E.F. Fritsch, and T. Maniatis, (1989), "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press: New York, NY.

Specification, page 20, lines 1-9. Thus, one of skill in the art is provided a direct citation to a prior art document that teaches the various ancillary and well-known protocols required to practice the invention. As noted in the above-quoted passage, these individual protocols are well-known to those skilled in the art and need not be described to the hypothetical person of ordinary skill in the art. This hypothetical practitioner, to whom the disclosure is directed, can clearly be imputed to have knowledge of all of these basic workhorse techniques of the molecular biologist.

Declaration of Periannan Senapathy:

In the first Office Action, the Office asked for additional evidence of the operability of the present invention. Applicant therefore submits for the Examiner's consideration the attached Rule 132 Declaration of inventor Periannan Senapathy, Exhibit C. The Declaration, which was prepared earlier in response to an Office Action in another of Applicant's patent applications, is admittedly not exactly on point, but provides ample evidence that the description contained in the subject patent application enables the claims as they are presently worded.

Apparently, Dr. Senapathy's Declaration either was not included with Applicant's earlier response or was misplaced by the Office. In reviewing his files, Applicant's undersigned counsel noticed that the earlier-submitted Declaration was an exact copy of Dr. Senapathy's Declaration as submitted in the other application, and thus had the same header. Thus, it seems quite likely that the Office directed the Declaration to the other application. The Declaration submitted with this response is also an exact copy of the first Declration, with the exception that the header information has been changed to reflect the serial number, filing date, title, examiner, and group art unit of the present application.

Paragraph 2 of his Declaration specifically states that Dr. Senapathy either conducted the experiments contained in his Declaration personally, or that they were carried out under his direction and supervision.

Paragraph 3 of the Declaration presents a brief overview of how an appropriate annealing temperature for a PCR run is selected based upon the average Tm of the two primers. Higher temperatures are more stringent, meaning the higher the temperature of annealing, the more specific the PCR run generally is. Dr. Senapathy also notes in paragraph 3 that the PCR methodology is, by its very nature, empirical, and must be adjusted for each new reaction.

At paragraph 5 of his Declaration, Dr. Senapathy introduces an experiment to determine the primer concentration necessary to amplify specifically a target DNA using a plurality of primers having a fixed portion and a randomized portion.

At paragraph 6, the experimental set-up is described. The template DNA is plasmid DNA (pGEM) and two control primers are described. A plurality of primers having 6 positions of fixed sequence and 8 positions randomized was then constructed. A PCR reaction using this plurality of primers was then conducted at 60°C (a very standard temperature) at various concentrations. The results are discussed in paragraph 7 and Figure 1 of the Declaration. The legend to Figure 1 of the Declaration recites all of the cycling parameters used in this reaction. As noted in paragraph 7, this reaction showed that the ideal concentration for the plurality of primers was 10X.

Paragraph 8 of the Declaration introduces a new experiment in which the number of fixed nucleotides within the plurality of primers was varied and using a much longer piece of template DNA (*E. coli* genomic DNA) (see paragraph 9). As noted in paragraph 9, three separate regions from the E. coli genome were chosen for amplification. Five primers for each of the three regions were constructed: 1) a first, fixed primer; 2) a second, fixed primer; 3) a plurality of partly-fixed second primers with 8 nucleotides fixed and 8 nucleotides fully randomized; 4) a plurality of partly-fixed second primers with 6 nucleotides fixed and 10 nucleotides fully randomized; and 5) a plurality of partly-fixed second primers with 5 nucleotides fixed and 11 nucleotides fully randomized (see paragraph 10).

Paragraph 11 of Dr. Senapathy's Declaration describes the concentration ranges tested, while paragraph 12 presents the exact nature of the five different primer sets for each of the three regions to be amplified specifically.

The purpose of this experiment is to demonstrate that the claimed process works using convention temperatures of annealing. As noted in paragraph 13 of the Declaration, the temperature of reannealing was raised gradually within the normal range used in the PCR protocol. The first experiment was conducted at an annealing temperature (Tm) within the normal range for all the three sets of experiments, namely 60° C. This temperature, however, is fairly stringent for the experiment with Region 3 (where the average Tm = 61°C), but not so stringent for Regions 1 and 2 (where the average Tm = 65.5° C and 62.2° C, respectively). The DNA from each of the three regions were PCR amplified in separate experiments with whole *E. coli* DNA as template. For each region, the PCR amplification between the first fixed primer and the second fixed primer was tested as a positive control.

The results are discussed in paragraph 14 of Dr. Senapathy's Declaration and in the attached Figure 2. Note that the particulars of the PCR runs are included in the legend to Figure 2 of the Declaration. In short, the results show that the expected band appears in the gel, with no significant non-specific amplification products. Paragraph 14, also notes, as is stated in the specification, that even if bands other than the expected band occur in the PCR products, sequencing would be done using the first primer, which is specific only to the particular DNA band amplified from the first primer location in the target DNA. Thus, the sequencing reaction should produce only the sequence of the specific expected DNA fragment.

This is, in fact, the case, as demonstrated by the sequencing reaction presented in paragraphs 16-18 of Dr. Senapathy's Declaration and the sequencing gels shown in Figure 3 of the Declaration. Most convincing from this experiment is that the expected sequence was obtained using all three different pluralities of partially-fixed, partially-random primers. All three pluralities yielded the same sequence data as the positive control. Clearly, this reaction convincingly demonstrates that the process as described and claimed, can, in fact, yield highly accurate, precise, reproducible, and useful sequencing data. Paragraph 18 of the Declaration specifically states that these results show that the presence of non-specific amplification products in the PCR products does not affect sequencing of the specifically-amplified fragment from the target region.

Beginning at paragraph 20 of his Declaration, Dr. Senapathy introduces another set of experiments designed to demonstrate the effect of primer concentration on the amplification

and sequencing reactions. In paragraph 21, the Declaration states that the experiment was conducted with the PCR amplifications carried out with and without increasing the concentration of the randomized second primer, using a DNA template of 12.5 KB. The object of this experiment is to show that increasing the concentration of the primers results in an increased amount of the specific amplification products. The results, discussed in paragraphs 21 and 22 and inFigures 6A and 6B, show that the primer with 6 random nucleotides at the 5' end (10-fold excess) and the primer with 8 random nucleotides at the 5' end (120-fold excess) amplified the target DNA fragment. The primer with 10 random nucleotides at a 250-fold excess, however, did not amplify the product, indicating that a higher concentration of the primer was needed in this reaction. Dr. Senapathy notes that these results demonstrate that target-specific amplification can be obtained by increasing the concentration of the plurality of partially-fixed, partially-randomized primers.

The sequencing experiment presented at paragraphs 23-25, and the sequencing gels shown in Figures 5A-5D of Dr. Senapathy's Declaration show that the amplification products produced by the reaction are sufficiently pure to be sequenced. Here, as noted in paragraph 25, a series of sequencing runs were performed using various pluralities of primer sets, including sets with 2, 4, 6, and 8, randomized base pairs. The specific primer sets are described in paragraph 25 of the Declaration and the results are depicted, quite convincingly, in the sequencer tracings shown in Figures 5A-5D.

The Declaration concludes with a final experiment, in paragraph 26, showing that the strength of the sequencing signals is inversely proportional to the number of random base pairs in the primer plurality. Here, in a fashion similar to the reaction described immediately above, a series of sequencing runs was performed with primer pluralities having 2, 4, 6, 8, and 10 randomized base pairs. The results show that the peak intensity was reduced as the number of randomized base pairs was increased. Nevertheless, even an 8-fixed/8 random primer was able to generate the correct sequencing data.

Clearly, in light of the objective scientific data presented in Dr. Senapathy's Declaration, data which was generated using the information specifically provided in the specification of the subject application, the specification enables the invention as claimed, in

full satisfaction of 35 USC §112, first paragraph. It is therefore respectfully requested that this rejection now be withdrawn.

Rejection of Claims 1-29 Under 35 USC §112, Second Paragraph:

This rejection is believed to have been rendered moot by appropriate amendment to Claims 1, 12, and 19. As amended, Claims, 12, and 19, the only independent claims in the application, now positively require that the various primers result in the specific amplification of a desired target nucleic acid.

In light of the amendment to Claims 1, 12, and 19, it is respectfully submitted that the rejection of Claims 1-29 under 35 USC §112, second paragraph, has been overcome. Withdrawal of the rejection is respectfully requested.

CONCLUSION

Applicant respectfully submits that the application is now in condition for allowance. Early notification of such action is earnestly solicited.

Respectfully submitted,

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Signature: Maura Layte

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. Serial No.: 09/431,451 Group Art Unit: 1650

Filing Date: November 1, 1999 Examiner: Sisson, B.

Applicant: Senapathy, P. Attorney Docket No.: 34623.005

Title: METHOD FOR AMPLIFYING SEQUENCES FROM UNKNOWN DNA

"MARKED UP" CLAIMS AS AMENDED, 37 CFR §1.121(c)(1)(ii)

1. (TWICE-AMENDED) A method of amplifying desired regions of nucleic acid from a nucleic acid template comprising:
   (a) providing a plurality of first PCR primers, each first primer having a region of fixed nucleotide sequence identical or complementary to a consensus sequence of interest and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence;
   (b) providing a plurality of second PCR primers, each second primer having a region of arbitrary, yet fixed nucleotide sequence and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence; and then
   (c) amplifying the nucleic acid template via the PCR using the plurality of first PCR primers and the plurality of second PCR primers[; whereby] under conditions wherein a subset of the plurality first primers binds to the consensus sequence of interest substantially wherever it occurs in the template, and a subset of the plurality of second primers binds to the template at locations removed from the first primers such that DNA regions flanked by the first primer and the second primer are specifically amplified.

12. (TWICE-AMENDED) A method of amplifying exons from a DNA template comprising:
   (a) providing a plurality of first PCR primers, each first primer having a region of fixed nucleotide sequence identical or complementary to a consensus sequence of a 3' splice region and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence;
   (b) providing a plurality of second PCR primers, each second primer having a region of fixed nucleotide sequence reversely complementary to a consensus sequence of a 5' splice region and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence; and then
   (c) amplifying the DNA template via the PCR using the plurality of first PCR primers and the plurality of second PCR primers[; whereby] under conditions

wherein a subset of the plurality first primers binds to a sequence reversely complementary to the 3' splice consensus sequence substantially wherever it occurs in the template, and a subset of the plurality of second primers binds to the 5' splice consensus sequence substantially wherever it occurs in the template, such that exons flanked by the first primer and the second primer are specifically amplified.

19. (TWICE-AMENDED) A method of amplifying regions flanking a consensus sequence in a nucleic acid template of totally or partially unknown sequence comprising:

(a) providing a plurality of first PCR primers, each first primer having a region of fixed nucleotide sequence identical or complementary to a consensus sequence of interest and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence;

(b) providing a plurality of second PCR primers, each second primer having a region of arbitrary, yet fixed nucleotide sequence and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence; then

(c) amplifying the nucleic acid template via the PCR using the plurality of first PCR primers and the plurality of second PCR primers[; whereby] under conditions wherein a subset of the plurality first primers binds to the consensus sequence of interest substantially wherever it occurs in the template, and a subset of the plurality of second primers binds to the template at locations removed from the first primers such that DNA regions flanked by the first primer and the second primer are specifically amplified; then

(d) incorporating the amplified nucleic acid of step (c) into a library;

(e) sequencing a portion of amplified nucleic acid from a particular clone from the library of step (d) and providing a third PCR primer of unique sequence which will prime PCR amplification from the sequenced portion of DNA;

(f) providing a plurality of fourth PCR primers, each fourth primer having a region of arbitrary, yet fixed nucleotide sequence and a region of randomized nucleotide sequence located 5' to, 3' to, anywhere within, or flanking the region of fixed nucleotide sequence; and then

(g) amplifying the nucleic acid present in the template via the PCR using the third PCR primer and the plurality of fourth PCR primers[; whereby] under conditions wherein the third primer binds to the sequenced portion of nucleic acid from step (e), and a subset of the plurality of fourth primers binds to the template at locations removed from the third primers such that DNA regions flanked by the third primer and the fourth primer are specifically amplified.